Journal of Forensic and Legal Medicine 20 (2013) 655–658

Contents lists available at SciVerse ScienceDirect

Journal of Forensic and Legal Medicine

journal homepage: www.elsevier.com/locate/jflm

Original communication

Validated UPLC-MS/MS assay for the determination of synthetic phosphodiesterase type-5 inhibitors in postmortem blood samples

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ARTICLE INFO

Article history:
Received 30 April 2012
Received in revised form
16 January 2013
Accepted 2 March 2013
Available online 24 April 2013

Keywords:
Sildenafil
Vardenafil
Tadalafil
UPLC-MS/MS

ABSTRACT

The use of synthetic phosphodiesterase type 5 (PDE-5) inhibitors for the treatment of erectile dysfunction: sildenafil citrate (Viagra®), tadalafil (Cialis®) and vardenafil hydrochloride (Levitra®) has increased dramatically over the past 2 years. These substances are prescription drugs and must be used under medical supervision. However, they can easily be obtained over the internet from illegal sites, being a potential for a threat to public health.

The development of an electrospray ionisation (ESI) ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) procedure for the simultaneous identification and quantification of three PDE5 inhibitors in blood samples was desired.

Samples were prepared using Oasis® HLB solid-phase cartridges (3 cc, 60 mg) and chromatographic separation was achieved on an Acquity UPLC® HSS T3 (100 × 2.1 mm i.d., 1.8 μm particles) column with a gradient mobile phase of 0.1% formic acid and acetonitrile at a 0.5 mL/min flow rate.

Quantification was achieved by multiple reaction monitoring (MRM) of two transitions per compound: m/z 475.1 > 58 e m/z 475.1 > 311.1 for sildenafil; m/z 389.9 > 267.9 e m/z 389.9 > 134.8 for tadalafil and m/z 489 > 71.9 e m/z 489 > 150.9 for vardenafil. Zolpidem-d6 (m/z 314.5 > 235.3) was used as the internal standard.

Calibration curves were linear over the concentration range of 5–1000 ng/mL, with a coefficient of determination better than 0.997. The lower limits of detection and quantification for these substances were ≤3 ng/mL and ≤8 ng/mL, respectively. The method showed a satisfactory sensitivity, precision, accuracy, recovery and selectivity.

A rapid, selective and sensitive UPLC-MS/MS method using solid-phase extraction was developed for the simultaneous determination and quantification of sildenafil, vardenafil and tadalafil in blood samples.

1. Introduction

Erectile dysfunction (ED) is the persistent inability to achieve and maintain an erection adequate for satisfactory sexual performance. Sexual stimulation induces nitricoxide (NO) release from endothelial cells and nonadrenergic and noncholinergic neurons. NO activates the cellular enzyme guanylate cyclase, which cleaves guanosine triphosphate into cyclic guanosine monophosphate (cGMP). cGMP in turn activates a series of downstream G proteins, which collectively lead to a decline in intracellular calcium content and subsequent smooth muscle relaxation. With muscular relaxation, there is a dilation of the cavernous arteries and corporal sinusoids of the penis, leading to enhancement of blood flow. Phosphodiesterase type 5 (PDE-5) is the enzyme primarily responsible for the hydrolysis of cGMP in the penis. Inhibition of PDE-5 leads to persistent vasodilation and maintenance of penile blood flow by sustaining a high level of cGMP in the cavernous tissues and arteries. Synthetic PDE-5 inhibitors are currently used in the treatment of male ED. Sildenafil citrate (*Viagra*®), tadalafil (*Cialis*®) and vardenafil hydrochloride (*Levitra*®) all inhibit PDE-5 at the level of corpus cavernous[1,2] with different onset of action, bioavailability (oral bioavailability of sildenafil averages 41%; vardenafil 15% and tadalafil 80%) and pharmacokinetic profiles.[3–6]

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http://dx.doi.org/10.1016/j.jflm.2013.03.002

However, tadalafil is innovative due to its longer half-life (17.5 and proven efficacy rates after 36 h) and highest selectivity.[1,6,7]

Because of its increasing popularity and potential side effects, the need for a procedure to the simultaneous determination of sildenafil, vardenatil and tadalafil in biological samples is becoming increasingly important.

Gas chromatography–mass spectrometry (GC/MS),[8,9] high-performance liquid chromatography (HPLC),[10–14] liquid chromatography–mass spectrometry (LC/MS)[15–19] as well as liquid chromatography–tandem mass spectrometry (LC/MS/MS)[20–28] methods have been reported for the determination of PDE-5 inhibitors in biological samples. The UPLC-MS/MS method provides a shorter runtime and sharper peak shape, which improves sensitivity and reduces potential interference by matrix components. Witjes et al.,[29] published a validated UPLC-MS/MS method for the analysis of sildenafil and its metabolite in plasma.

We present the validation results of a method for simultaneous assay of sildenafil, tadalafil and vardenafil in blood samples after solid-phase extraction, using UPLC-MS/MS. To our knowledge, this is the first sensitive and selective UPLC-MS/MS method to simultaneous quantify three PDE-5 inhibitors in blood samples.

2. Materials and methods

2.1. Chemicals and materials

Sildenafil (99.7%) and zolpidem-d6 (99%), used as internal standard (IS), 1 mg/mL and 100 μg/mL ampoules, respectively, were obtained from Cerilliant (Round Rock, TX, USA); tadalafil (not less than 98%) powder was supplied by TLC PharmaChem Inc. (Concord, Canada) and vardenafil hydrochloride (99.9%) powder was kindly provided by Bayer HealthCare (Wuppertal, Germany). Formic acid extra pure (98–100%) and methanol analytical grade were purchased from Merck (Darmstadt, Germany). Acetonitrile and water LC-MS-grade were from Merck (Darmstadt, Germany). The mobile phase was filtered through a 0.20 μm filter (Schleicher & Schuell) and degassed in an ultrasonic bath for 15 min prior to use. Oasis HLB® SPE Cartridges (60 mg, 3 mL), used for sample preparation, were from Waters (Waters® Corporation, Milford, MA, USA), and were used on a solid-phase system Vac Elut EPS 24 from Varian (Harbor City, CA, USA). Standard and deuterated stock solutions of 1 and 0.1 mg/mL in methanol were stored at −20 °C. For each compound two separate stock solutions of 1 and 0.1 mg/mL were prepared in methanol and identified as calibration and quality control (QC), respectively.

Working standard solutions were prepared by dilution of stock solutions to appropriate concentrations. All solutions were stored at −20 °C.

2.2. Blood samples

Pooled blank human blood was obtained from the Portuguese Blood Institute (Coimbra, Portugal) and used for selectivity studies and to create spiked blood samples for the validation of the analytical procedure. All blood samples were collected into plastic tubes containing 1% potassium fluoride and Na_2EDTA-oxalate and stored at −20 °C prior to analysis.

Qualitative analysis was performed in central blood samples and peripheral blood was selected for the corresponding quantitative analysis.

2.3. Instrumentation

An ACQUITY Ultra Performance LC system (Waters® Corporation, Milford, MA, USA), composed of a binary solvent delivery manager, a thermostatted autosampler and column over compartment was used. Separation was performed on an Acquity UPLC® HSS T3 column, 2.1 × 100 mm, packed with 1.8 μm particles, which was maintained at 35 °C.

The mobile phase, consisting of acetonitrile (solvent A) and 0.1% formic acid in water (solvent B), was delivered at a flow rate of 0.50 mL/min. The gradient program was as follows: initial 75% B for the first 1 min, then gradient elution was performed by changing the mobile phase from 75% to 40% B between 1 and 6 min and after this time, reversion of the mobile phase to 75% B. At the end of this sequence the column was equilibrated under initial conditions for 1.0 min. The autosampler temperature was set at 15 °C. The system was equipped with strong and weak wash solution reservoirs.

Detection was carried out using an Acquity™ TQD tandem-quadrupole MS equipped with a Z-spray electrospray ionization (ESI) source (Waters® Corporation, Milford, MA, USA).

The mass spectrometer was operated in positive multiple reaction mode (MRM) mode. Parameters were as follows: capillary voltage (3.0 kV), extractor voltage (3.0 V), RF lens voltage (0.1 V), source temperature (150 °C), desolvation temperature (450 °C),

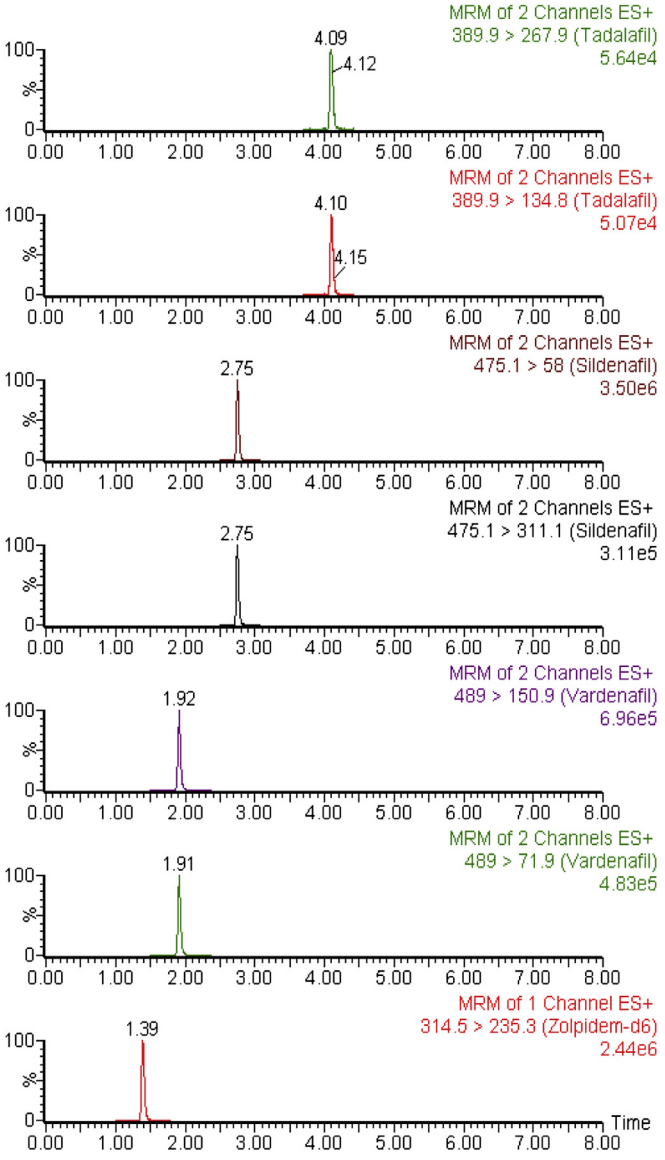

Fig. 1. UPLC-MS/MS chromatograms with two MRM transitions monitored simultaneously for each PDE-5 inhibitors in blood samples.

Table 1
Retention time (RT), optimized ion transitions and collision energy, limits and linearity results.

Analyte	RT (min)	DP (V)	Targetion transition (*m/z*)	CE (eV)	Qualifierion transition (m/z)	CE (eV)	Limits (ng/mL)		Linearity	r^2
							LD	LQ		
Sildenafil	2.75	54	475.1 > 58	44	475.1 > 311.1	32	3	8	$Y = 0.0091x + 0.0044$	0.998
Tadalafil	4.10	36	389.9 > 267.9	20	389.9 > 134.8	14	1	4	$Y = 0.0003x + 0.0011$	0.997
Vardenafil	1.92	52	489 > 150.9	42	489 > 71.9	54	3	8	$Y = 0.0007x - 0.0039$	0.997
Zolpidem-d6	1.39	56	314.5 > 235.3	38	–	–	–	–	–	–

DP declustering potencial, CE collision energy.

cone gas flow rate (0 L/h), desolvation gas flow rate (750 L/h), multiplier voltage (650 V) and collision gas flow (0.15 mL/min).

The analytes were detected by ion transitions from protonated molecule ions to fragment ions in MRM mode. For each analyte, the most intense ion transition (target ion) was used for quantitation and the second one (qualifier ion) to confirm its identification (Fig. 1). Retention time, declustering potential, collision energy and MRM transitions for the analytes are listed in Table 1.

All aspects of system operation and data acquisition were controlled using Masslynx™ v4.1 software (Waters® Corporation, Milford, MA, USA) with automated data processing using the TargetLynx™ Application Manager (Waters® Corporation, Milford, MA, USA). IntelliStart™ software (Waters® Corporation, Milford, MA, USA) was used to control the fluidics device to infuse solutions for tuning the MS.

Argon was used as collision gas, and nitrogen was used as the nebulising and desolvation gas.

2.4. Sample preparation

The QC and the calibration samples were prepared by spiking drug-free blood samples with methanolic working standard solutions.

A 0.5 mL aliquot of blood sample was spiked with 50 μL of IS (zolpidem-d6 was used due to the lack of adequate reference standard) at a concentration of 1 μg/mL and 2 mL of water was added. The mixture was vortex mixed for 30 s following centrifugation at 2000 × *g* for 5 min and loaded into HLB cartridge, which had been conditioned with 2 mL methanol followed by 2 mL water. The washing of the cartridges was carried out with 5% (v/v) aqueous methanol (2 mL). Vacuum was applied on the solid-phase system for 10 min to dry the solvent. The analytes were eluted with 2 mL methanol. The elution was evaporated to dryness using a Turbo Vap LV evaporator (Caliper, Hopkinton, MA, USA) under a gentle stream of nitrogen at 40 °C. The dried extract was then reconstituted with 150 μL of phase mobile (25:75, v/v acetonitrile - 0.1% formic acid in water) and 10 μL was injected into the UPLC-MS/MS system in full loop mode.

2.5. Method validation

The UPLC-MS/MS procedure for the quantification of sildenafil, tadalafil and vardenafil in blood was validated according to internationally accepted recommendations.[30–33]

For the determination of selectivity and specificity, ten pools of drug-free blood samples, nine consisting of a mixture of four postmortem blood samples and one prepared with three antemortem blood samples, were extracted as described previously without the addition of IS. In addition, a blank sample with IS were analyzed for peaks deriving from IS and interfering with the detection of the analytes. All chromatograms were free of background interference (0% false positives and <10% false negatives). The assay was found to be selective and specificity for all tested compounds, no interfering peaks were observed.

To determine linearity, blank blood samples were spiked at ten different concentration levels and then analyzed. Calibration curves were linear over the concentration range of 5–1000 ng/mL, with coefficients of determination (r^2) better than 0.997 and intercepts were all close to zero and not statistically significant (Table 1).

Limit of detection (LD) and limit of quantification (LQ) were determined at low concentrations between 0.5 and 20 ng/mL, using six equidistant calibration points. The calibration curves were fitted linearly ($r^2 \geq 0.998$) and LD and LQ were calculated using $(3.3 \times S_{y/x})/b$ and $(10 \times S_{y/x})/b$, respectively, were $S_{y/x}$ is calibration curve residual standard deviation and b is calibration curve slope. LD and LQ of PDE-5 are listed in Table 1.

QC samples (QC low and QC medium) were prepared at concentrations described in Table 2. Three samples of each QC concentration were measured over a period of five consecutive days. Daily calibration curves were used to calculate the concentration of the QCs.

Precision data for within-day (repeatability) and time-different intermediate precision (combination of within and between-day effect) of the method were calculated using one-way analysis of variance (ANOVA) with the grouping-variable day. Repeatability was estimated in a single assay by extraction and analysis of QC samples with five replicates at each concentration. Repeatability and intermediate precision values expressed as RSD % were lower than 15%, which is within the acceptance criteria for validating analytical methods (Table 2). Accuracy is expressed as mean % deviation from the nominal concentration of the quality controls. Mean accuracy results were not be significantly different from 100%.

The extraction efficiency (recovery) was assessed by comparison of the peak area ratios obtained for the three PDE-5 inhibitors after extraction from blood with those obtained without extraction. The experiment was conducted at two QC concentrations and all samples were processed in triplicate. The recovery values are listed in Table 2.

Table 2
Validation data of repeatability, intermediate precision, accuracy and recovery of PDE-5 inhibitors in blood samples.

Analyte	QC Sample (ng/mL)	Measured conc. (ng/mL)	Repeatability CV (%)	Intermediate precision CV (%)	Accuracy (%) ($n = 15$)	Recovery (%) ($n = 3$)
Sildenafil	100	112.0	4.8	9.3	112.0	87.6
	500	514.4	2.2	2	102.9	91.9
Tadalafil	100	115.7	5.7	12.3	115.7	51.8
	500	516.6	1.8	2.2	103.1	85.2
Vardenafil	100	116.0	7.7	12.3	116.0	68.1
	500	512.0	2.3	1.7	102.4	80.7

Since the procedure proved to be sensitive, selective and reproducible, the method developed was successfully implemented to analyze postmortem blood samples in routine forensic investigation.

The authors present two cases of postmortem blood samples, in which PDE-5 inhibitors were detected: one case, a 54 year-old male, with tadalafil (72 ng/mL) and another case, a 26 year-old male, with sildenafil (144 ng/mL). In both cases blood concentrations of PDE-5 inhibitors were detected in therapeutic levels.[3,34]

3. Conclusion

We present the validation results of a method for simultaneous assay of sildenafil, tadalafil and vardenafil in blood samples after solid-phase extraction, using UPLC-MS/MS, being a selective UPLC-MS/MS method that simultaneous quantify three PDE-5 inhibitors in blood samples, important in forensic toxicology routine analysis.

Ethical approval
None.

Funding
None.

Conflict of interest
None.

Acknowledgments

The authors want to thank Bayer Portugal S.A. (Quality Assurance) and Bayer HealthCare AG Business Group Pharma for its valuable contribution by providing a standard of vardenafil hydrochloride.

References

1. Aversa A. Systemic and metabolic effects of PDE5-inhibitor drugs. *World J Diabetes* 2010;**15**:3–7.
2. Burnett AL, Lowenstein CJ, Bredt DS, Chang TS, Snyder SH. Nitric oxide: a physiologic mediator of penile erection. *Science* 1992;**257**:401–3.
3. Baselt RC. *Disposition of toxic drugs and chemicals in man*. 7th ed. Biomedical Publications; 2004. 1024–1025, 1178, 1227.
4. Aversa A, Bruzziches R, Pili M, Spera G. Phosphodiesterase 5 inhibitors in the treatment of erectile dysfunction. *Curr Pharm Des* 2006;**12**:3467–84.
5. Moffat AC, Osselton MD, Widdop B. *Clarke's analysis of drugs and poisons in pharmaceuticals, body fluids, and postmortem material*. 3th ed. The Pharmaceutical Press; 20041559–60.
6. Washington SL, Shindel AW. A once-daily dose of tadalafil for erectile dysfunction: compliance and efficacy. *Drug Des Dev Ant Ther* 2010;**4**:159–71.
7. Forgue ST, Patterson BE, Bedding AW, Payne CD, Philips DL, Wrishko RE, et al. Tadalafil pharmacokinetics in healthy subjects. *Br J Clin Pharmacol* 2006;**61**: 280–8.
8. Papoutsis I, Nikolaou P, Athanaselis S, Pistos C, Maravelias C, Spiliopoulou C. A fully validated method for the determination of vardenafil in blood using gas chromatography/mass spectrometry. *J. Mass Spectrom* 2011;**46**:71–6.
9. Strano-Rossi S, Anzillotti L, Torre X, Botre F. A gas chromatography-mass spectrometry method for the determination of sildenafil, vardenafil and tadalafil and their metabolites in human urine. *Rapid Commun Mass Spectrom* 2010;**24**:1697–706.
10. Al-Ghazawin M, Tutunji M, AbuRuz S. Simultaneous determination of sildenafil and n-desmethyl sildenafil in human plasma by high-performance liquid chromatography method using electrochemical detection with application to a pharmacokinetic study. *J Pharm Biomed Anal* 2007;**43**:613–8.
11. Cooper JDH, Muirhead DC, Taylor JE, Baker PR. Development of an assay for the simultaneous determination of sildenafil (Viagra) and its metabolite (UK-103,320) using automated sequential trace enrichment of dialysates and high-performance liquid chromatography. *J Chromatogr B* 1997;**701**:87–95.
12. Rodríguez J, Berzas JJ, Castañeda G, Rodrıguez N. Determination of sildenafil citrate (Viagra) and its metabolite (UK-103,320) by square-wave and adsorptive stripping square-wave voltammetry. Total determination in biological samples. *Talanta* 2004;**62**:427–32.
13. Cheng C, Chou C. Determination of tadalafil in small volumes of plasma by high-performance liquid chromatography with UV detection. *J Chromatogr B* 2005;**822**:278–84.
14. Carlucci G, Palumbo P, Iuliani P, Palumbo G. Development of a method for the determination of vardenafil in human plasma by high performance liquid chromatography with UV detection. *Biomed Chromatogr* 2009;**23**:759–63.
15. Lee MY, Min DI. Determination of sildenafil citrate in plasma by high-performance liquid chromatography and a case for the potential interaction of grapefruit juice with sildenafil citrate. *Ther Drug Monit* 2001;**23**:21–6.
16. Pistos C, Papoutsi I, Dona A, Stefanidou M, Athanaselis S, Maravelias C, et al. Off-line HPLC method combined to LC–MS for the determination of sildenafil and its active metabolite in post-mortem human blood according to confirmation criteria. *Forensic Sci Int* 2008;**178**:192–8.
17. Tracqui A, Ludes B. HPLC-MS for the determination of sildenafil citrate (Viagra) in biological fluids. Application to the salivary excretion of sildenafil after oral intake. *J Anal Toxicol* 2003;**27**:88–94.
18. Drumestre-Toulet V, Cirimele V, Gromb S, Beloussoff T, Lavault D, Ludes B, et al. Last performance with Viagra®: post-mortem identification of sildenafil and its metabolites in biological specimens including hair sample. *Forensic Sci Int* 2002;**126**:71–6.
19. Tracqui A, Miras A, Tabib A, Raul JS, Ludes B, Malicier D. Fatal overdosage with sildenafil citrate (Viagra): first report and review of literature. *Human & Experim Toxic* 2002;**21**:623–9.
20. Wang Y, Wang J, Cui Y, Fawcett J, Gu J. Liquid chromatographic–tandem mass spectrometric method for the quantitation of sildenafil in human plasma. *J Chromatogr B* 2005;**828**:118–21.
21. Eerkes A, Addison T, Naidong W. Simultaneous assay of sildenafil and desmethylsildenafil in human plasma using liquid chromatography–tandem mass spectrometry on silica column with aqueous–organic mobile phase. *J Chromatogr B* 2002;**768**:277–84.
22. Kim J, Ji H, Kim S, Lee H, Lee S, Kim D, et al. Simultaneous determination of sildenafil and its metabolite UK-103,320 in human plasma using liquid chromatography-tandem mass spectrometry. *J Pharm Biomed Anal* 2003;**32**: 317–22.
23. Lewis JR, Johnson RD, Blank LC. Quantitative determination of sildenafil (Viagra) and its metabolite (UK-103,320) in fluid and tissue specimens obtained from six aviation fatalities. *J Anal Toxicol* 2006;**30**:14–20.
24. Weinmann W, Bohnert M, Wiedemann A, Renz M, Lehmann N, Pollak S. Postmortem detection and identification of sildenafil (Viagra) and its metabolites by LC/MS and LC/MS/MS. *Int J Leg Med* 2001;**114**:252–8.
25. Ku HY, Shon JH, Liu KH, Shin JG, Bae SK. Liquid chromatography/tandem mass spectrometry method for the simultaneous determination of vardenafil and its major metabolite, N-desethylvardenafil, in human plasma: application to a pharmacokinetic study. *J Chromatogr B* 2009;**877**:95–100.
26. Johnson RD, Lewis RJ, Angier MK. The postmortem distribution of vardenafil (levitra®) in a aviation accident victim with an unusually high blood concentration. *J Anal Toxicol* 2007;**31**:328–33.
27. Lake ST, Altman PM, Vaisman J, Addison RS. Validated LC–MS/MS assay for the quantitative determination of vardenafil in human plasma and its application to a pharmacokinetic study. *Biomed Chromatogr* 2010;**24**:846–51.
28. Ramakrishna NVS, Vishwottam KN, Puran S, Koteshwara M, Manoj S, Santosh M, et al. Quantitation of tadalafil in human plasma by liquid chromatography–tandem mass spectrometry with electrospray ionization. *J Chromatogr B* 2004;**809**:243–9.
29. Witjes BC, Ahsman MJ, Nagel BC, Tibboelb D, Mathot RA. Simultaneous assay of sildenafil and desmethylsildenafil in neonatal plasma by ultra-performance liquid chromatography–tandem mass spectrometry. *Biomed Chromatogr* 2010;**24**:180–5.
30. Ermet J. Validation in pharmaceutical analysis. Part I: an integrated approach. *J Pharm Biomed Anal* 2001;**24**:755–67.
31. Peters FT, Maurer HH. Bioanalytical method validation and its implications for forensic and clinical toxicology. A review. *Accred Qual Assur* 2002;**7**:441–9.
32. Peters FT, Drummer OH, Musshoff F. Validation of new methods. *Forensic Sci Int* 2007;**165**:216–24.
33. Hartmann C, Smeyers-Verbeke J, Massart DL, McDowall RD. Validation of bioanalytical chromatographic methods. *J Pharm Biomed Anal* 1998;**17**: 193–218.
34. TIAFT The International Association of Forensic Toxicologists. *Reference blood level list of therapeutic and toxic substances*. TIAFT, http://www.tiaft.org/tmembers/ttv/ttv_ab.htsml; 2005.